CELL
APPLICATIONS, INC.

General Instructions for Culturing

Human Pre-Screened Skeletal Muscle Cells-COPD (S-HSkMC-COPD)

Be sure to wear face protection mask and gloves when retrieving cryovials from the liquid nitrogen storage tank. The dramatic temperature change from the tank to the room could cause any trapped liquid nitrogen in the cryovials to burst and cause injury.

Open all the packages immediately upon arrival and examine each component for shipping damage. Notify Cell Applications, Inc. or your distributor immediately if there is any problem.

I. STORAGE

A. CRYOPRESERVED VIALS (S150COPD -05)
Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.

B. PROLIFERATING FLASKS (S151COPD-25, -75)

1. Examine under a microscope to check if all the cells are attached to the bottom of the flask. If not, notify CAI or your distributor immediately.
2. Decontaminate the exterior of the cell culture flask with 70% alcohol.
3. Place the sealed flask in a 37°C, 5% CO_2 humidified incubator for 2 hours as shipped.
4. In a sterile Biological Safety Cabinet, open the cap of the flask very slowly and carefully.
5. Remove the Transport Medium by aspiration. Add fresh Growth Medium: 5 ml for a T-25 flask and 15 ml for a T-75 flask.
6. Place the flask in a 37°C, 5% CO_2 humidified incubator with loosened cap to allow gas exchange.
7. Change medium every other day.

C. GROWTH MEDIUM (151-500)
Store the Growth Medium at 4°C in the dark immediately upon arrival.

D. SUBCULTURE REAGENT KIT (090K)
Store at -20°C immediately upon arrival.
Store at 4°C after thawing.

E. DIFFERENTIATION MEDIUM (151D-250)
Store the Differentiation Medium at 4°C in the dark immediately upon arrival.

F. COLLAGEN SOLUTION (125-50)
Store at 4°C in the dark immediately upon arrival.

II. PREPARATION FOR CULTURING

1. Make sure the Class II Biological Safety Cabinet, with HEPA filtered laminar airflow, is in proper working condition.
2. Clean the Biological Safety Cabinet with 70% alcohol to ensure it is sterile.
4. Make sure all serological pipettes, pipette tips and reagent solutions are sterile.
5. Follow the standard sterilization technique and safety rules:
   a. Do not pipette with mouth.
   b. Always wear gloves and safety glasses when working with human cells even though all the strains have been tested negative for HIV, Hepatitis B and Hepatitis C.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING S-HSkMC-COPD

A. PREPARING CELL CULTURE FLASKS FOR CULTURING S-HSkMC-COPD

1. Take the Skeletal Muscle Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 15 ml of Skeletal Muscle Cell Growth Medium* into a T-75 flask.

* Keep the medium to surface area ratio at 1 ml per 5 cm^2. For example,
5-7.5 ml for a T-25 flask or a 60 mm tissue culture dish.
15-20 ml for a T-75 flask or a 100 mm tissue culture dish.

B. THAWING AND PLATING S-HSkMC-COPD

1. Remove the cryopreserved vial of S-HSkMC-COPD from the liquid nitrogen storage tank using proper protection for your eyes and hands.
2. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.
3. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath and watch the vial closely during the thawing process.
4. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.
5. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
6. Remove the vial cap carefully. Do not touch the rim of the cap or the vial.
7. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette. Be careful not to pipette too vigorously as to cause foaming.
8. Pipette the cell suspension (1ml) from the vial into the T-75 flask containing 15 ml of Skeletal Muscle Cell Growth Medium.
9. Cap the flask and rock gently to evenly distribute the cells.

10. Place the T-75 flask in a 37ºC, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.
11. Change to fresh Skeletal Muscle Cell Growth Medium after 24 hours or overnight to remove all traces of DMSO.
12. Change Skeletal Muscle Cell Growth Medium every other day until the cells reach 60% confluent.
13. Double the Skeletal Muscle Cell Growth Medium volume when the culture is >60% confluent or for weekend feedings.
14. Subculture the cells when the S-HSkMC-COPD culture reaches 85-95% confluent.

IV. SUBCULTURING S-HSkMC-COPD

A. PREPARING SUBCULTURE REAGENTS

1. Remove the Subculture Reagent Kit from the -20°C freezer and thaw overnight in a refrigerator.
2. Make sure all the subculture reagents are thawed. Swirl each bottle gently several times to form homogeneous solutions.
3. Store all the subculture reagents at 4°C for future use. The activity of Trypsin/EDTA Solution will be stable for 2 weeks when stored at 4°C.
4. Aliquot Trypsin/EDTA solution and store the unused portion at -20°C if only portion of the Trypsin/EDTA is needed.

B. PREPARING CULTURE FLASK

1. Take the Skeletal Muscle Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 35 ml of Skeletal Muscle Cell Growth Medium to a T-175 flask (to be used in Section IV C Step 15).

C. SUBCULTURING S-HSkMC-COPD

Trypsinize Cells at Room Temperature. Do Not Warm Any Reagents to 37°C.

1. Remove the medium from culture flasks by aspiration.
2. Wash the monolayer of cells with HBSS and remove the solution by aspiration.
3. Pipette 5 ml of Trypsin/EDTA Solution into the T-75 flask. Rock the flask gently to ensure the solution covers all the cells.
4. Remove 4 ml of the solution immediately.
5. Re-cap the flask tightly and monitor the trypsinization progress at room temperature under an inverted microscope. It usually takes about 2 to 5 minutes for the cells to become rounded. The cells may not be completely round during trypsinization and some cells may maintain some processes even though they are loosened from the culture surface.
6. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.
7. Pipette 5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.
8. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.
9. Rinse the flask with an additional 5 ml of Trypsin Neutralizing Solution and transfer the solution into the same conical tube.
10. Examine the T-75 flask under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9.
11. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells.
12. Aspirate the supernatant from the tube without disturbing the cell pellet.
13. Flick the tip of the conical tube with your finger to loosen the cell pellet.
14. Resuspend the cells in 5 ml of Skeletal Muscle Cell Growth Medium by gently pipetting the cells to break up the clumps.
15. Count the cells with a hemocytometer or cell counter. Inoculate at 10,000 cells per cm^2 for rapid growth, or at 5,000 cells per cm^2 for regular subculturing.

V. DIFFERENTIATING S-HSkMC-COPD

A. PREPARING CELL CULTURE WARE

1. Swirl the Collagen Solution bottle a few times to form a homogenous solution.
2. Decontaminate the bottle with 70% alcohol in a sterile hood.
3. Determine the appropriate format to seed S-HSkMC-COPD for the experiment e.g., plates or flasks.
4. Add Collagen Solution to tissue culture ware at coating concentration of 1 ml per $10cm^2$ surface area of culture ware for 30 minutes at 37ºC or 2 hours (overnight is OK) at room temperature.
5. Remove the Collagen Solution by aspiration in a sterile hood.
6. Wash the Collagen coated surface two times with PBS.
7. The coated flask can be used immediately or stored at 4ºC for up to one month.

B. SEEDING S-HSkMC-COPD FOR DIFFERENTIATION

1. Seed S-HSkMC-COPD in Skeletal Muscle Growth Medium at cell density of 20,000/cm^2 for fetal S-HSkMC-COPD and 32,000/cm^2 for adult S-HSkMC-COPD.
2. Start to induce differentiation the next day, follow instructions in Section V C.

C. DIFFERENTIATING S-HSkMC-COPD TO MYOTUBES

1. Pre-equilibrate Skeletal Differentiation Medium (amount needed for differentiation) in a 37ºC, 5% CO_2 humidified incubator for 2 hours.
2. Remove growth medium from culture tissue ware by aspiration. Do not allow cells to dry during medium changes.
3. Add the appropriate volume of Skeletal Muscle Differentiation Medium, at 1ml/$5cm^2$.
4. Incubate cell in a 37ºC, 5% CO_2 humidified incubator in the Skeletal Muscle Differentiation Medium.
5. Change to fresh Skeletal Muscle Differentiation Medium every other day.
6. Multinucleal myotubes will form typically within 3-4 days for fetal skeletal muscle cells and 6-7 days for adult skeletal muscle cells.

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